NEUROKININ RECEPTOR EXPRESSION IN THE LYMPHATIC SYSTEM

A Senior Scholars Thesis

by

PARTH VIJAY KHADE

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Biomedical Engineering

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Approved by:

Research Advisor: Dave C. Zawieja
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Neurokinin Receptor Expression in the Lymphatic System. (April 2009)

Parth Vijay Khade
Department of Biomedical Engineering
Texas A&M University

Research Advisor: Dr. Dave C. Zawieja
Department of Systems Biology and Translational Medicine

The lymphatic system plays important roles in the maintenance of body fluid homeostasis, lipid absorption and immune function. These functions are accomplished by its contractile activities that result in the flow of lymph. One of the important modulators of lymphatic function and lymph flow is Substance P (SP), a neuropeptide of the tachykinin family that is associated with sensory innervation of lymphoid tissue. It mediates its functions through G protein coupled receptors NK1R and NK3R. However, it is unclear which specific receptors are involved in SP action in the lymphatic system. This project specifically focused on the expression pattern of the two neurokinin receptors in different rat lymphatic tissues and lymphatic cell lines measured by western blots. The mean ratio of NK1R/GAPDH was found to be 1.766 ± 0.120 in rat mesenteric lymphatic muscle cell lines and 1.443 ± 0.264 in rat thoracic duct muscle cell lines. The mean ratio of NK3R/GAPDH was found to be 0.371 ± 0.051 in rat mesenteric lymphatic muscle cell lines and 0.733 ± 0.063 in rat thoracic duct muscle cell lines. This molecular information will be useful in our future studies to determine the specific mechanisms of SP on lymphatics and to help elucidate the role of SP in the modulation of lymphatic function.

DEDICATION

To my loving parents Vijay and Vinita, and to my brother Dhruv, for all of their love and support.

ACKNOWLEDGMENTS

I would first like to thank my research advisor Dr. Dave Zawieja. Dr. Zawieja has given me an invaluable amount of support and knowledge in the field of lymphatic biology research during my time at Texas A&M University. He has continually given me the opportunity to conduct research in his laboratory, starting from my first semester at Texas A&M, something which I am very thankful for. He has taken a great deal of personal time to explain to me important concepts and theories in lymphatic biology. Dr. Zawieja has served as a role model for many aspiring young researchers, and I am greatly indebted to him for his dedication and for all of the opportunities he has given me.

I would also like to thank Dr. Sanjukta Chakraborty for working with me in the lab while conducting this project. Dr. Chakraborty has devoted time and effort to helping me understand fundamental concepts and has played an integral role in helping me become the researcher that I am today. Her guidance and patience have provided me with an opportunity to succeed in the field of research. I would also like to thank Dr. Muthuchamy for his insight on receptor expression measured by western blots, which helped me when writing my thesis. I would also like to thank Scott, Pat, and Andrea for their help in the lab.

Finally, I would like to thank my parents, Vijay and Vinita, as well as my brother Dhruv, for all of their continuous love and support.

NOMENCLATURE

SP	Substance P
NK1R	Neurokinin 1 Receptor
NK3R	Neurokinin 3 Receptor
RTDMC	Rat Thoracic Duct Muscle Cell
RMLMC	Rat Mesenteric Lymphatic Muscle Cell
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GPCR	G-Protein Coupled Receptor

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CHAPTER I

INTRODUCTION

The lymphatic system

The lymphatic system is a specialized part of the cardiovascular system that is intimately involved in the maintenance of body fluid balance, protein homeostasis and immune function. It is one of the primary systems that returns fluids and proteins from interstitial tissue to blood in the circulatory system. The lymphatic system is comprised of the conducting system, which includes lymph capillaries, vessels, and right thoracic ducts, as well as lymphoid tissue, which is comprised of lymphocytes and various white blood cells contained in connective tissue through which lymph passes. This tissue includes the thymus, bone marrow, and lymph nodes. The conducting system is responsible for the transportation of lymph through lymphatic channels, while lymphoid tissue is responsible for immune functions in response to infections and the spreading of tumors. Lymph is formed when interstitial fluid enters the lymph capillaries. Lymph components interact with blood components via their respective sinuses within the lymph nodes. The lymphatic system has no central pump, but uses spontaneous contraction of lymphatic vessels, to lead to lymph flow. The contraction of lymph vessels is influenced by neural, physical, and humoral factors. These factors act on the lymphatic system to regulate lymph flow under pathological and physiological conditions. A dysfunctional lymphatic circulation can result in a wide range of clinical disorders including edema, altered lymphocyte circulation, depressed immune function and impaired lipid metabolism (1). It is known that lymphatic function is critical to the development and resolution of edema; however there are only few efficacious therapies, as well as a nearly complete lack of medicinal treatment options for lymphatic dysfunction.

This thesis follows the style of The American Journal of Pathology.

This is mainly due to the poor understanding of contractile activity of lymphatic muscle cells that is essential for lymph flow (2,3). Thus investigation of the mechanisms generating or regulating lymphatic muscle contractile function is important to better understand lymphatic function and obtain deeper insights into the pathogenesis and the effective treatment of lymphedema (4).

Previous functional studies have shown that Substance P stimulates contraction of rat mesenteric lymphatic vessels. Since lymph flow is an important factor during inflammation and immune function, it is important to study the effects that SP might have on it. SP is found and released from C-sensory nerve fibers which surround lymphatic vessels in the body. SP secreted during inflammation by lymphatic vessels could be responsible for lymphocyte and immune activation. At low concentrations, SP also enhances lymphatic contractility and pump efficiency. In quiescent vessels, this stimulation of lymphatic contraction frequency causes an active pumping state, resulting in an increased lymph flow (5). SP is also able to modify lymphatic pump function by reducing the resting lymphatic diameter (EDD), thus causing an increase in smooth muscle cell tone. It is proposed that SP release is graded and is thus able to modify lymphatic vessel function and hence lymph flow in a concentration dependent manner (6). The increased contraction of vessels is important because increased lymph flow can be vital to minimize the formation of edema.

Substance P

Substance P is a mammalian 11-amino acid neuropeptide that belongs to a family of structurally related peptides called tachykinins, which include neurokinin A and neurokinin B. SP, NKA, and NKB all have a particular carboxyl-terminal motif in common (Phe-X-Gly-Leu-MET-NH_2) and are derived from preprotachykinins through

proteolytic processing (7). SP is the major mediator of inflammatory responses and affects multiple aspects of immune cell function, and is often associated with cells of the lymphoid tissue. Some of the biological responses of Substance P include motor control, contraction of smooth muscle, vasodilation and sensory perception. Substance P can act both directly and indirectly to elicit its effects on target tissue. For example, SP can directly stimulate endothelium-dependent nitric oxide production to increase vasodilation, or it can stimulate the release of cytokines and chemokines, which indirectly leads to vasodilation and increased permeability (6). SP is known to mediate its effects through neurokinin receptors (NK1R, NK2R, NK3R). These receptors are seven transmembrane G-protein coupled receptors that all bind SP albeit with varying affinities, with NK1R having the highest affinity for SP. NK1R and NK2R are localized to smooth muscle cells and to a few inflammatory cells. NK1R expression is also found in the muscular wall of submucosal blood vessels, enteric neurons and, in surface epithelial cells. NK3R is found throughout the brain (8).

Although SP displays the strongest affinity for the NK1R, NK2R emerges as the prime receptor for SP mediated signaling in esophageal smooth muscle (8). It is not clearly understood which specific neurokinin receptor plays a key role in lymphoid tissue, but it is suggested that SP maybe acting through a combination of these receptors in the lymphatic system (6). However there is no documented evidence on the expression of these receptors in the lymphatic tissue.

Signaling pathways of Substance P

There are many signaling pathways in the body which Substance P acts through to induce its effects. Studying these pathways is integral in determining treatments for related physiological conditions. One of the primary signaling pathway through which

Substance P acts on that has been studied is the MAP Kinase (MAPK) Pathway. This pathway is present in many different systems, for example – SP stimulates the expression of TNF-α mRNA in peritoneal mast cells through the P38 and JNK MAPK pathway (9). Substance P also acts in human astrocytomas and glioblastomas by stimulating NK1R to activate the MAPK pathway through the activation of PKC (10).

Substance P has also been shown to stimulate the synthesis of chemokines in pancreatic acinar cells by acting through the NF-kB pathway (11). NF-kB is a protein transcription factor that binds to DNA located within the intronic enhancer in B-cells and plasma cells. Chemokines are cytokines which illicit chemotaxis in surrounding cells, and are considered pro-inflammatory. These inflammatory mediators also play a role in pathogenesis. Substance P acts through the NK1 receptor to activate the NF-kB transcription factor and greatly increase the synthesis of CC chemokine MCP-1, CC chemokine MIP-1α, and CXC chemokine MIP-2. One chemokine in particular, MCP-1, has been shown to be released by acinar cells to mediate inflammation in acute pancreatitis. This is significant because a systemic inflammation response can result, which can lead to the dysfunction of multiple organs and even death (9).

Other mechanisms through which Substance P acts include the inositol phosphate and calcium signaling pathways. Substance P has been shown to produce excitatory and inhibitory effects in the neurons of the nucleus tractus solitarii in the central nervous system. Most of the time Substance P produces an excitatory effect however, through the mobilization of intracellular calcium, however Substance P has been shown to depress transmission of sensory input to bronchopulmonary neurons by acting at presynaptic NK1 receptors to decrease glutamate release (glutamate is the principal excitatory neurotransmitter at this). This is done by activating a calcium dependent potassium current which hyperpolarizes vagal afferent neurons. Overall, Substance P controls the

reflex regulation of respiratory function and depresses the frequency of eEPSCs (excitatory postsynaptic currents). Abnormalities in the regulation of respiratory function can lead to conditions such as bronchitis, asthma, and chronic obstructive pulmonary disease (12).

It has been shown that all three receptor subtypes (NK1R, NK2R, and NK3R) are present in the smooth muscle of the human airway. Although Substance P (which causes changes in airway contraction) mostly acts on NK1R, NK2R and NK3R have binding affinity for tachykinins neuropeptides similar to Substance P – Neurokinin A and Neurokinin B, respectively. All three of these receptors are able to induce the release of intracellular calcium from the sarcoplasmic reticulum of the human airway smooth muscle by coupling to G_q protein mediated inositol 1,4,5-triphosphate, also known as the IP3 receptor. IP3 mediates the initial elevation of calcium concentration. This, along with the sustained release phase of calcium, initiates contraction in the smooth muscle surrounding the airway. The changes in airway resistance are important when considering pharmacological treatments for respiratory diseases, including obstructive lung diseases (13).

Tachykinin signaling in esophageal smooth muscle has also been characterized. The primary neurokinin receptors (NK1R, NK2R, and NK3R) have been found to be located on esophageal smooth muscle cells. Tachykinins including SP primarily mediate their effects through the NK2 receptor in these cells. These effects include Ca^{2+} influx through L-type channels, Ca^{2+} release from intracellular stores, and activation of the I_{NSC} current. Thus, the presence of tachykinin signaling in the enteric nerves of the GI tract will cause circular and longitudinal contraction, which plays an integral role in mediating peristalsis (8).

CHAPTER II

METHODS

Tissue isolation

Frozen lymphatic thoracic, mesenteric, cervical and iliac tissues were available in the Sponsor's laboratory.

Cell culture

Rat thoracic duct lymphatic muscle cells (RTDMC) and rat mesenteric lymphatic muscle cells (RMLMC) isolated from rat thoracic and mesenteric tissue explants respectively were available in the laboratory. Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics. The cells were maintained in a 37^{o}C incubator with 10% CO_2. Maintenance of the cell cultures included washing, feeding, trypsinizing, splitting, and freezing of the cells. All activity involving cell cultures was conducted inside a hood to prevent contamination. RTDMC and RMLMC cells were initially plated and cultured on 35 mm dishes, grown to confluence, trypsinized, and then transferred subsequently to 60 100 mm dishes.

Washing cells

The cell cultures were washed daily to remove any debris/waste that had accumulated, so that the cells would be able to grow unhindered in a healthy environment. Old media was discarded, and cells were washed with DPBS (Dulbecco's Phosphate Buffered Saline).

Feeding cells

The cell cultures were fed with fresh media to obtain the appropriate nutrients for growth. The media used was DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum) and 1% triple antibiotics (to prevent bacterial growth). This complete media included different peptidases and enzymes necessary for cell culture growth that were not present in plain media (glucose only). After feeding, cell cultures were placed back inside a 37 ^{o}C incubator.

Trypsinizing cells

Cells were trypsinized once they became confluent. The cells were then split - part or all of the cell culture in one plate was transferred to another plate. Adding trypsin to the cell cultures allowed the cells to cleave from their substrate (the dish) and move freely. Cell cultures were trypsinized after being washed with DPBS. Once the buffer was removed from the dish, .25% EDTA-Trypsin was added. 800 uL was used for 35 mm plates, while 1 mL was used for 100 mm plates. The plates were rotated from side to side to ensure proper distribution of trpysin. Immediately after trypsin was added to the cultures, they were placed inside of the 37 ^{o}C incubator for 3 – 5 minutes as that is the optimal temperature for trypsin action. The cultures were then taken out and observed underneath a light microscope to ensure that the cells were no longer adhered to the plate. Next, approximately 4 mL of DMEM were added. The serum in the media was used to stop the action of trypsin.

Splitting cells

This step took place after DMEM had been added and thoroughly mixed with the trypsinized cell mixture. About half of the mixture was pipetted out and added to a new 100 mm plate.

Freezing cells

Some of the cell cultures were frozen back for later use. Freezing media was composed of 50% DMEM, 40% FBS, and 10% DMSO. First, old media was removed from the culture plates and the plate was then washed with 1 mL of DPBS. Cells were then trypsinized. After cells were completely trypsinized, 2 mL of DMEM were added to stop the effects of trypsin. Next, 3 more mL of plain media (glucose) were added. Cells were thoroughly mixed by pipetting up and down to ensure a homogenous suspension. The cells suspension was then transferred into 15 mL tubes. Each tube had 6 mL of media. Then, the tubes were centrifuged at 1000 rpm for 10 minutes. A pellet of the cells was formed at the bottom of each tube. The supernatant was discarded. Depending on the size of the pellet, 500 uL of freezing media was then added/tube to be frozen and the cell pellet was resuspended. This was then transferred into cryovials and stored -80 ^{o} C for 2-3 days and then subsequently transferred to liquid nitrogen.

Protein isolation

Protein was isolated from the different rat lymphatic tissues (thoracic, cervical, and mesenteric lymphatic duct) and RTDMC and RMLMCs. Cells were grown to confluence on 100 mm plates and then scraped using protein lysis buffer. Protein lysis buffer contained protease inhibitors to prevent protein degradation and ETDA-trypsin to cleave

cells from substrate. Cells and tissues were sonicated in an SDS protein lysis buffer. The samples were sonicated for 3 minutes and then placed on ice for 1 minute. This process was repeated 3 times. The samples were then boiled for 5 minutes. Protein samples were stored at -80 ^{o} C until future use.

Western blot

Western blotting is a useful technique for the identification and quantification of specific proteins in complex mixtures of proteins. The protein isolated from rat tissues and cell lines were separated on a 4-20% gradient SDS-polyacrylamide gel. Bio-Rad Precast Tris-HCL gels were used to separate proteins. The 12-well gel was 8.6 cm x 6.8 cm and comprised of 4% stacking gel (4-20% resolving gel). The gel was run in a Tris-Glycine buffer (10x). This was then transferred to a nitrocellulose membrane overnight. Protein transfer was verified by Ponceau S staining. The membrane was then soaked in TBS (Tris buffered saline) buffer to wash off Ponceau stain. The membrane was then blocked with 5% milk solution in TBS. The primary antibodies (NK1R, NK3R) were then added to the membrane at the appropriate dilution and incubated overnight at 4^{0}C. The membranes were then washed with TBS 3 times for 10 minutes each and the corresponding secondary antibody was added. Antibody binding was detected by an electrochemiluminescence method using the Pierce Detection system using an X-ray film. The blot was then stripped off and re-probed with another primary antibody as before. Protein expression analysis for each of the two receptors was done in five replicates. The blots were stripped off using immunopure IgG elution buffer and probed with a housekeeping gene GAPDH to check for equal loading. Results were expressed as mean±SEM.

CHAPTER III

RESULTS

Rat thoracic duct muscle cells and rat mesenteric lymphatic muscle cells were cultured and reached confluency in 5 - 7 days. To determine the concentration of NK1R and NK3R, RTDMC and RMLMC lysates which contained these protein receptors were isolated. Cell lysate was obtained through the use of protein lysis buffer from both RTDMC and RMLMC cultures. Protein lysate was obtained from 5 different RTDMC and RMLMC cultures, so that 5 different replicates could be tested. The purpose of using 5 different replicates from each cell line was to obtain more accurate statistical data when calculating average ratios.

SDS page

A western blot probing for alpha smooth muscle actin was first run to determine the optimal concentration of protein lysate to be loaded into the stacking gel for separation. Actin was used due to its prevalence in smooth muscle cells. It is a globular protein and has a molecular weight of 42 KDa. 5 wells were loaded with samples of Actin which increased in concentration by 5 μL. 5 μL, 10 μL, 15 μL, 20 μL, and 30 μL were loaded into consecutive wells of a SDS-polyacrylamide gel. The gel was run and transferred to a blot, which was verified through Ponceau S stain. A western blot was then run, which probed alpha smooth muscle actin with an actin primary antibody at a concentration of 1:10,000. A Mouse IgG secondary antibody of concentration 1:10,000 was then used to probe the actin primary antibody. The optimal concentration was determined to be 20 μL – it was at this concentration that clear, sharp bands of actin protein were shown. Thus, 20 μL of RTDMC and RMLMC protein lysate would be inserted into each well for the SDS-PAGE.

Since a 12 well stacking gel was used, all 5 replicates from each cell line were able to be loaded in alternating fashion into a single gel. The first well of the gel was loaded with a protein marker, the next 10 wells were loaded with alternating samples of RTDMC and RMLMC protein lysate replicates, and the last well was left empty. SDS-PAGE was run to separate all protein from the RTDMC and RMLMC lysate into separate bands. The progress of protein separation was tracked using glycerol dye. The SDS-PAGE ran to completion, that is, all protein was separated, in 45 minutes. The gel was transferred to a membrane overnight, and the resulting blot was stained with Ponceau S stain to verify protein transfer. The blot is shown below in figure 1.

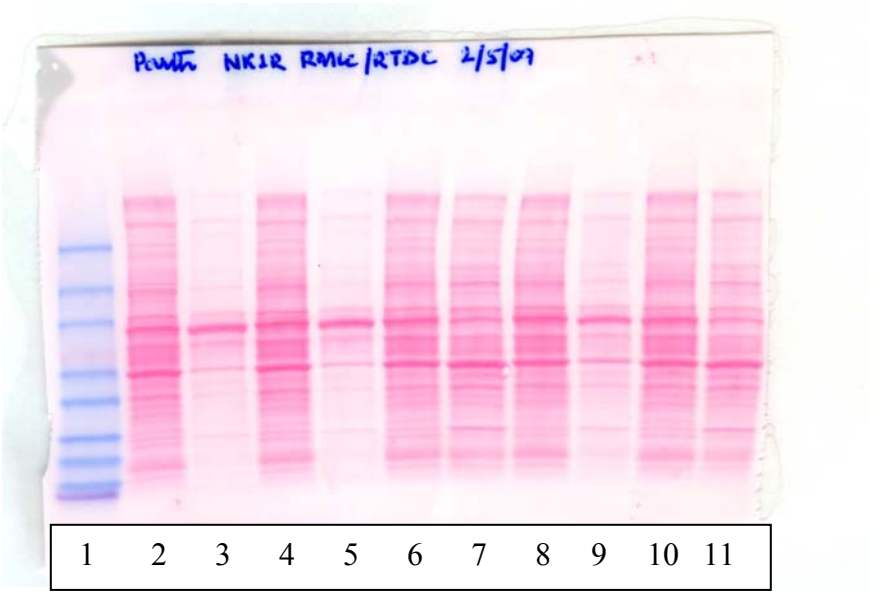

Figure 1. Ponceau S Stain of first RTDMC and RMLMC protein blot

The first well of this blot was loaded with 20 µL of marker protein. The blue-colored bands from the marker protein were used to indicate the molecular weights precisely so as to determine which bands corresponded to NK1R and NK3R proteins. The molecular weight for NK1R is 53 KDa and the molecular weight for NK3R is 66 KDa. The table

for this marker is shown on Table 1, with the proteins numbered from the top down on the blot.

Table 1. Molecular weights of protein marker

Band #	MW (KDa)
1	170.8
2	109.5
3	78.9
4	60.4
5	47.2
6	35.1
7	29.9
8	18.3
9	13.7
10	5.7

On this blot, the 2nd, 4th, 6th, 8th, and 10th wells were loaded with RTDMC samples. The 3rd, 5th, 7th, 9th, and 11th wells were loaded with RMLMC samples. Although each well was loaded with an equal amount of lysate, lanes 3, 5, and 9 seemed to contain a smaller amount of protein. When these lanes are compared to lanes 2, 4, 6, 7, 8, 10, and 11, a clear difference is noted in the amount of protein present. This smaller amount of RMLMC protein lysate also clearly corresponds to a smaller amount of NK1R and NK3R, as is indicated by the protein marker. Because of this, another gel was to run to obtain the necessary amount of samples for the proposed 5 replicates. However, all 5 wells loaded with RTDMC samples (2nd, 4th, 6th, 8th, and 10th wells) showed adequate amount of protein in equal concentrations, including NK1R and NK3R. Thus, the protein

samples of NK1R and NK3R found in those 5 wells would be able to be used when calculating the average ratio to GAPDH.

Another SDS-PAGE was run with alternating wells of RTDMC and RMLMC smooth muscle cell lysate to compensate for the smaller amount of protein lysate observed in wells 3, 5, and 9 in the blot from figure 1. 20 µL of protein lysate were again loaded into each well, with RTDMC and RMLMC samples alternating lanes. The gel was transferred to a membrane overnight, and the resulting blot was stained with Ponceau S stain to verify protein transfer. The new membrane is presented below in figure 2.

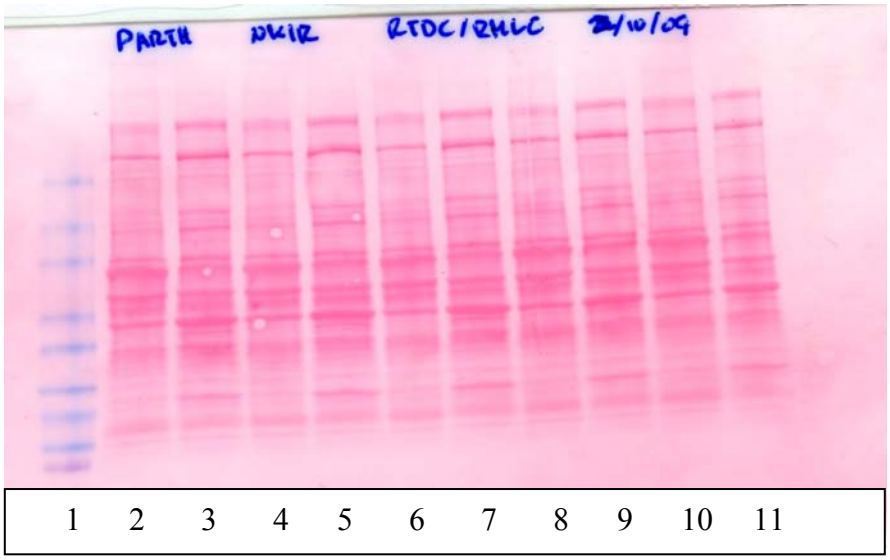

Figure 2. Ponceau S Stain of second RTDMC and RMLMC protein blot

This blot presented a more equal distribution of protein within each well. Also, the amount of separated protein now appeared consistent from well to well. This was important as each well was loaded with equal amounts of protein lysate, so an equal amount and distribution of protein would be expected.

Western blots

Now that two gels with an equal distribution of protein were obtained and transferred to their respective blots, the western blot protocol was started. Both blots were probed with primary antibodies for NK1R and NK3R. The concentration values for both primary and secondary antibodies for NK1R and NK3R had been previously standardized; these same values were used to probe the blots in this experiment.

The concentration of NK1R antibody was determined to be 1:1000 – 10 μL of primary antibody was put into 10 mL of 5% milk blocking solution. The secondary antibody used for NK1R was Rabbit IgG (immunoglobulin G). Its standardized concentration was determined to be 1/10,000 – 1 μL of secondary antibody was put into 10 mL of 5% milk blocking solution.

The concentration of NK3R antibody was determined to be 1:2000 – 5 μL of primary antibody was put into 10 mL of 5% milk blocking solution. . The secondary antibody used for NK3R was also Rabbit IgG (immunoglobulin G). Its standardized concentration was determined to be 1/8,000 – 1.25 μL of secondary antibody was put into 10 mL of 5% milk blocking solution.

The concentration of the GAPDH antibody was determined to be 1:1000 – 10 μL of primary antibody was put into 10 mL of 5% milk blocking solution. The secondary antibody used for GAPDH was also Rabbit IgG (immunoglobulin G). Its standardized concentration was determined to be 1/5,000 – 2 μL of secondary antibody was put into 10 mL of 5% milk blocking solution.

ECL detection

The membranes were probed for NK1R, NK3R, and GAPDH with primary and secondary antibodies using the western blot protocol, described previously in the methods section. Blots were stripped after each probing with an immunopore IgG elution buffer. Antibody binding was detected using the electrochemiluminescence method, and x-ray films of the western blots were developed using the Pierce Detection System. The results of the ECL detection are presented below in figures 3, 4, and 5.

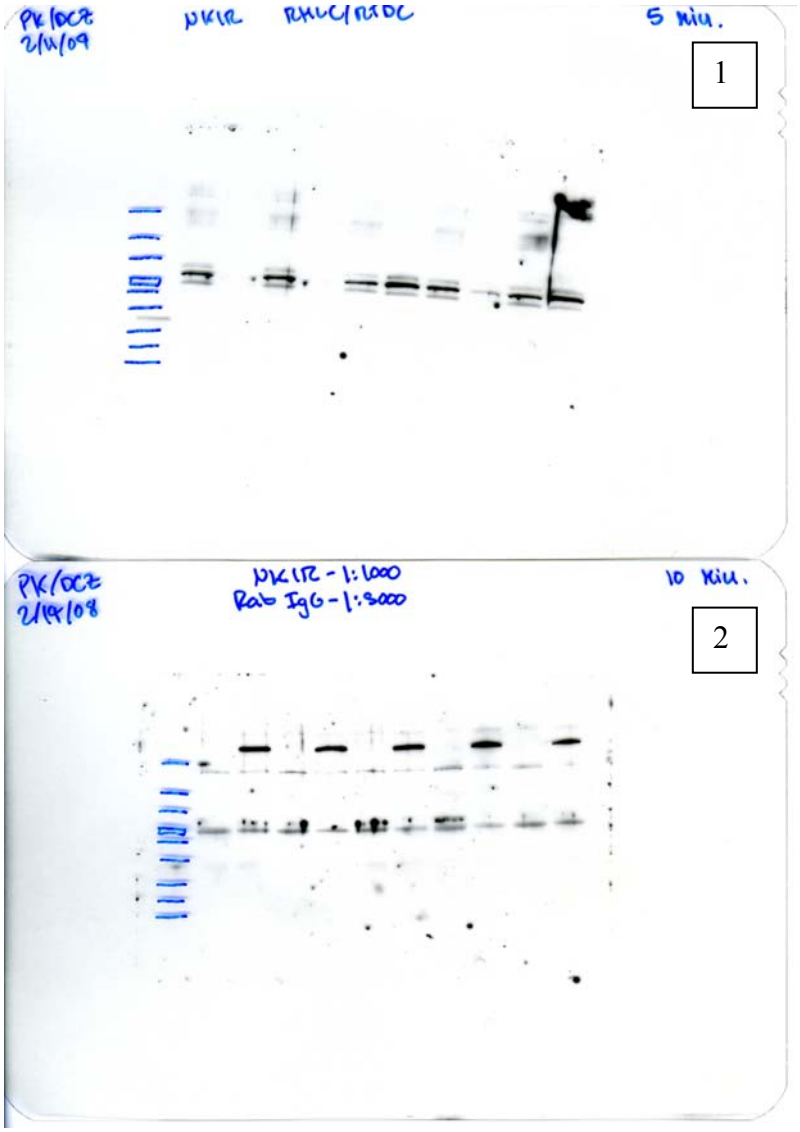

Fig. 3 NK1R western blot using ECL detection method

The images in figure 3 display the developed film of both 1st and 2nd blots detecting antibody binding to NK1R. Exposure to H_2O_2 and luminol was varied to give optimal density of bands with minimal background. It is interesting to note that in both blots NK1R and NK3R expression occurred in doublets. It is also interesting to note that antibody binding to a protein of greater molecular weight than 170.8 KDa was detected in only in RMLMC lanes of the second blot. Optimal time for exposure of the 1st blot

was found to be 5 minutes. Optimal time for exposure of the 2nd blot was found to be 10 minutes.

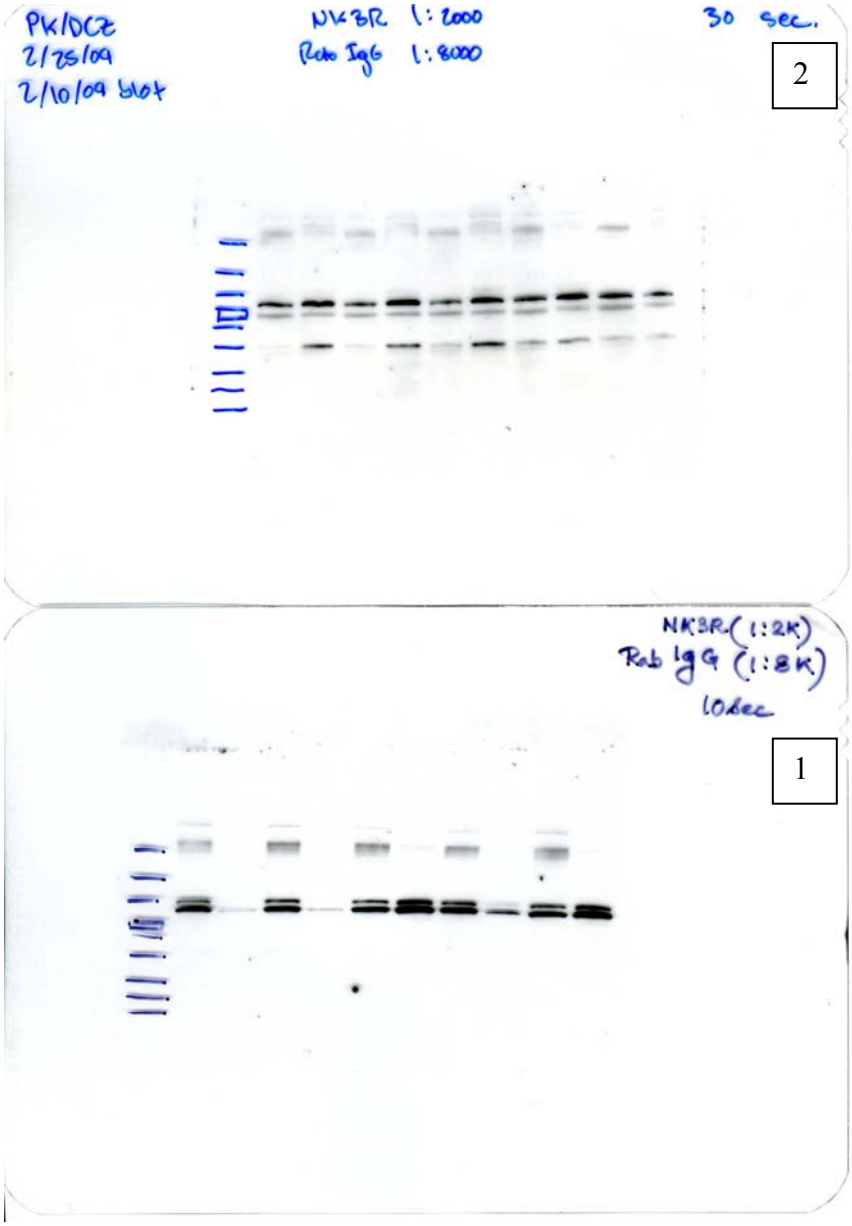

Fig. 4 NK3R western blot using ECL detection method

The images in figure 4 display the developed film of both 1st and 2nd blots detecting antibody binding to NK3R. Optimal time for exposure of the 1st blot was found to be 10 seconds. Optimal time for exposure of the 2nd blot was found to be 30 seconds. Once again, NK1R and NK3R expression occurred in doublets.

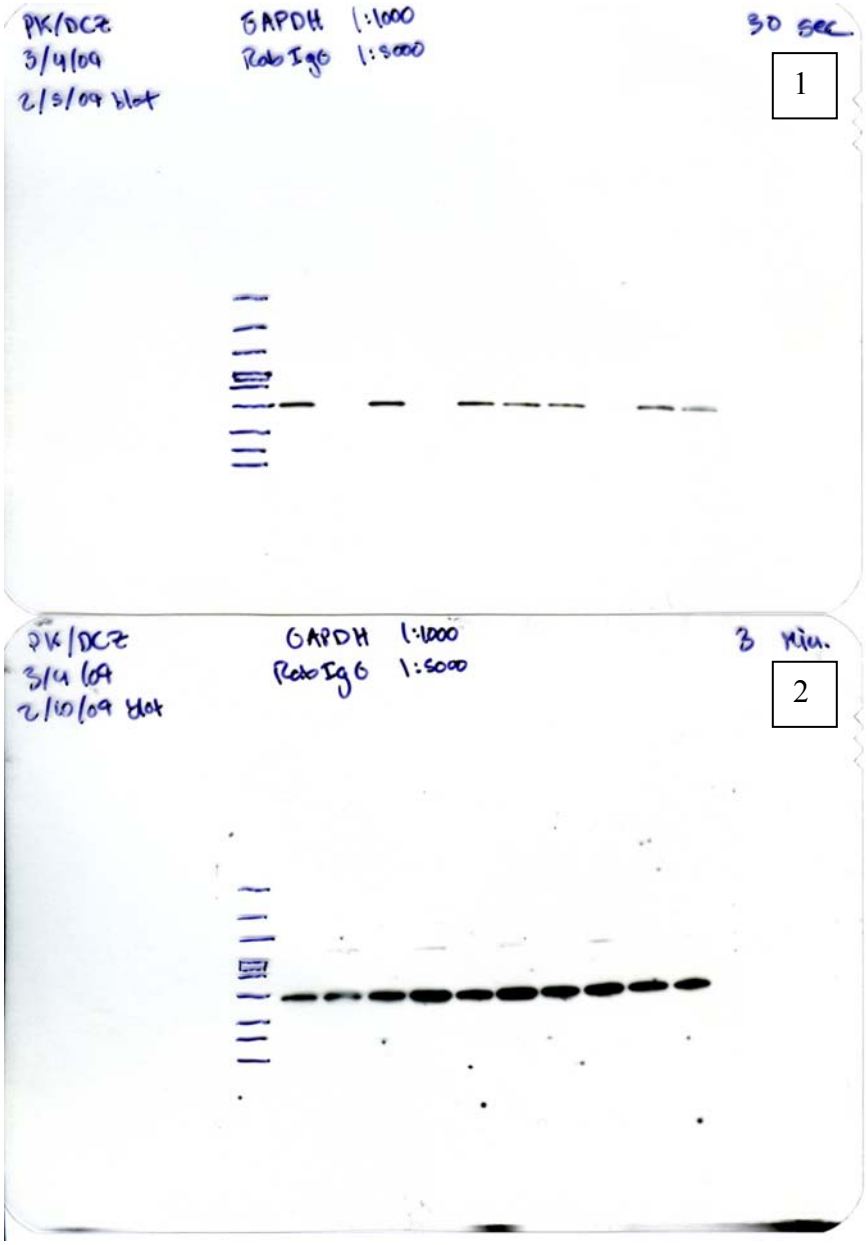

Fig. 5 GAPDH western blot using ECL detection method

The images in figure 5 display the developed film of both 1st and 2nd blots detecting antibody binding to GAPDH. Both blots were probed with the housekeeping gene GAPDH to check for equal loading. Optimal time for exposure of the 1st blot was found to be 30 seconds. Optimal time for exposure of the 2nd blot was found to be 3 minutes.

Ratios of adjusted volume

As the ultimate purpose of this project was to determine the protein expression pattern of NK1R and NK3R receptors in different lymphatic muscle cell lines, the relative expression ratios of protein receptors to those of control housekeeping gene GAPDH were determined using the Bio-Rad software program Quantity 1. Quantity 1 was able to analyze a wide variety of biological samples, including chemiluminescent samples. Image samples could be magnified, rotated, and resized. Total and average quantities, as well as determine relative and actual amounts of protein were able to be measured.

In the tables below, each index represents a protein pool sample from a different plate of cells. The adjusted volume (INT*mm^2) of the receptors NK1R and NK3R from both rat thoracic duct muscle cells and rat mesenteric lymphatic muscle cells was measured using Quantity 1, along with the respective adjusted volume of GAPDH. The adjusted volumes of both receptor proteins and GAPDH were recorded. These values were then used to calculate the expression ratio of the protein receptors to those of GAPDH (NK1R/GAPDH and NK3R/GAPDH). The mean ratio, standard deviation, and the standard error of mean of all ratios were also calculated and recorded.

Table 2. Ratios of adjusted volume of NK1R to GAPDH in RTDMC

RTDMC - NK1R							
Index	Name	Adj. Vol. NK1R	Adj. Vol. GAPDH	Ratio of NK1R to GAPDH	Mean Ratio	Standard Deviation	SEM
1	U1	1801.903	1585.929	1.136182	1.44295041	0.591197766	0.264392
2	U2	1577.919	1613.129	0.978173			
3	U3	1338.951	1345.776	0.994929			
4	U4	1925.942	829.9217	2.320631			
5	U5	1807.722	1012.822	1.784838			

Table 2 above contains data for the expression of NK1R in RTDMC replicates. The index column lists all replicates in the order obtained. 5 replicates were obtained for the NK1R protein receptor in the RTDMC line. The adjusted volume of NK1R ranged from 1339 INT*mm^2 – 1926 INT*mm^2. The adjusted volume of GAPDH for these replicates ranged from 830 INT*mm^2 to 1613 INT*mm^2. Ratios were calculated by finding the quotient values between the adjusted volumes of NK1R and GAPDH. The average ratio was calculated to find the best representative ratio of NK1R between all RTDMC replicates. The standard deviation and standard error of mean were also calculated to check for consistency – the relatively high standard deviation and standard error of mean indicate that further tests might be beneficial to obtain more consistent results.

Table 3. Ratios of adjusted volume of NK1R to GAPDH in RMLMC

RMLMC - NK1R							
Index	Name	Adj. Vol. NK1R	Adj. Vol. GAPDH	Ratio of NK1R to GAPDH	Mean Ratio	Standard Deviation	SEM
1	U1	1313.573	797.9397	1.64620594	1.76581797	0.169156954	0.119612
2	U2	1208.126	640.7695	1.885429999			

Table 3 above contains data for the expression of NK1R in RMLMC replicates. The index column lists all replicates in the order obtained. Only 2 replicates were obtained for the NK1R protein receptor in the RMLMC line due to insufficient optical density and slightly excessive background on the developed x-ray film. The adjusted volume of NK1R ranged from 1208 INT*mm^2 to 1313 INT*mm^2. The adjusted volume of GAPDH for these replicates ranged from 641 INT*mm^2 to 798 INT*mm^2. Ratios were calculated by finding the quotient values between the adjusted volumes of NK1R and GAPDH. The average ratio was calculated to find the best representative ratio of NK1R between all RMLMC replicates. Although only two replicates were present, the standard deviation and standard error of mean were still calculated to check for consistency.

Table 4. Ratios of adjusted volume of NK3R to GAPDH in RTDMC

RTDMC – NK3R							
Index	Name	Adj. Vol. NK3R	Adj. Vol. GAPDH	Ratio of NK3R to GAPDH	Mean Ratio	Standard Deviation	SEM
1	U1	813.3941	1340.899	0.606603428	0.73278743	0.188121409	0.062707
2	U2	797.1263	1056.784	0.754294599			
3	U3	614.6596	983.8624	0.624741476			
4	U4	919.2005	850.354	1.08096213			
5	U5	859.6411	927.3261	0.92701055			
6	U6	813.8922	1613.129	0.504542516			
7	U7	719.9644	1345.776	0.534980884			
8	U8	657.293	829.9217	0.791993975			
9	U9	779.8294	1012.822	0.769957303			

Table 4 above contains data for the expression of NK3R in RTDMC replicates. The index column lists all replicates in the order obtained. 9 replicates were obtained for the NK3R protein receptor in the RTDMC line. The adjusted volume of NK3R ranged from 615 INT*mm^2 to 919 INT*mm^2. The adjusted volume of GAPDH for these replicates ranged from 641 INT*mm^2 to 798 INT*mm^2. The average ratio was calculated to find

the best representative ratio of NK3R between all RTDMC replicates. The standard deviation and standard error of mean were also calculated to check for consistency. The relatively low standard error of mean for NK3R in this cell line indicates consistent fairly consistent ratios.

Table 5. Ratios of adjusted volume of NK1R to GAPDH in RMLMC

RMLMC – NK3R							
Index	Name	Adj. Vol. NK1R	Adj. Vol. GAPDH	Ratio of NK3R to GAPDH	Mean Ratio	Standard Deviation	SEM
1	U1	1304.138	2306.054	0.565527829	0.37076312	0.115114754	0.051481
2	U2	1230.915	3337.563	0.368806615			
3	U3	957.5141	3323.107	0.28813823			
4	U4	1101.01	3158.178	0.348621856			
5	U5	807.8421	2857.382	0.282721093			

Table 5 above contains data for the expression of NK3R in RMLMC replicates. The index column lists all replicates in the order obtained. 5 replicates were obtained for the NK3R protein receptor in the RMLMC line. The adjusted volume of NK3R ranged from 808 INT*mm^2 to 1304 INT*mm^2. The adjusted volume of GAPDH for these replicates ranged from 2306 INT*mm^2 to 3338 INT*mm^2. The average ratio was calculated to find the best representative ratio of NK3R between all RMLMC replicates. The standard deviation and standard error of mean were also calculated to check for consistency. The relatively low standard error of mean for NK3R in this cell line indicates consistent ratios.

Plotted results

The results from the tables above are expressed as mean ratio ± SEM in figures 6 and 7 below. Two graphs are presented – the first for NK1R, and the second for NK3R. Both RMLMC and RTDMC cell lines are included in the graph for each receptor. The average ratios for each cell line are plotted as bars while the standard errors of mean are included as error bars for an accurate representation.

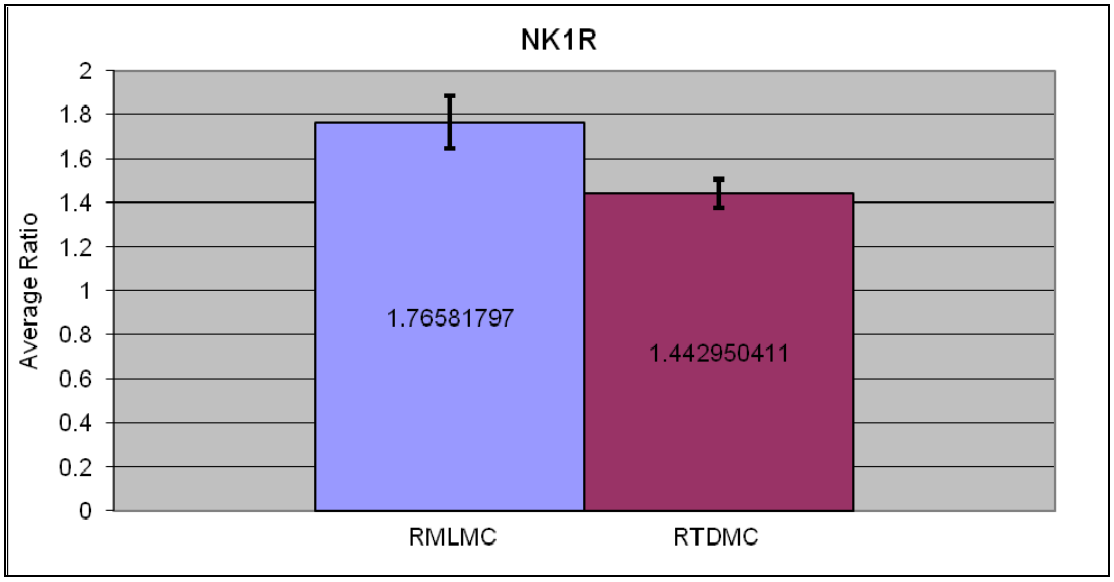

Figure 6. Mean ratio ± SEM of NK1R

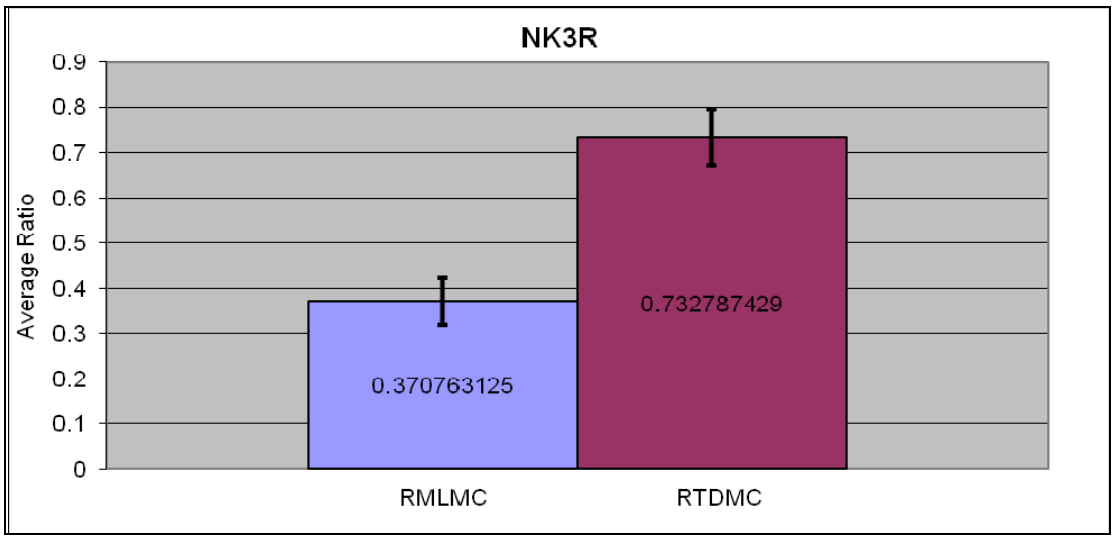

Figure 7. Mean ratio ± SEM of NK3R

CHAPTER IV

SUMMARY AND CONCLUSIONS

In conclusion, NK1R was shown to have slightly greater expression in RMLMC lines than in RTDMC lines, and NK3R was shown to have significantly greater expression in RTDMC lines than in RMLMC lines. The specific expression ratio of NK1R/GAPDH was found to be 1.766 ± 0.120 in RMLMC lines and 1.443 ± 0.264 in RTDMC lines. The specific expression ratio of NK3R/GAPDH was found to be 0.371 ± 0.051 in rat mesenteric lymphatic muscle cell lines and 0.733 ± 0.063 in rat thoracic duct muscle cell lines. As RMLMC cell lines were isolated from rat mesenteric lymphatic tissue beds, and RTDMC lines were isolated from thoracic duct tissue beds, these ratios could also indicate the slightly greater presence of NK1R in rat mesenteric lymphatic muscle tissues, and significantly greater presence of NK3R in rat thoracic duct muscle tissues. It will be important in future studies to investigate the expression of these receptors in tissues as well.

It was also interesting to note that NK1R and NK3R were found to be expressed in doublets. This could occur because both NK1R and NK3R could have similar structural and conformational sites of phosphorylation. The NKRs could also be expressed as doublets due to N-glycosylation of residue sites which enhances GPCR stability in the plasma membrane and possibly protects receptors from proteolytic degradation (14). NK1R contains two N-linked glycosylation sites, Asn-14 and Asn-18 (15). This is significant as both NK1R and NK3R are G-protein coupled receptors (GPCRs) which take part in several different pathways, including the Map Kinase pathway as well as the inositol phosphate and calcium signaling pathways. As the primary function of GPCRs is to transduce external stimuli into intracellular signals, the greater stability of these receptors could play a further role in their signal transduction. N-glycosylated receptors

have been shown to display greater functional cell surface expression and more rapid internalization upon ligand binding. Finally, it has been suggested that kinase activity and self-dimerization depends on protein N-glycosylation (16).

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CONTACT INFORMATION

Name:	Parth Vijay Khade
Professional Address:	c/o Dr. Dave C. Zawieja Department of Systems Biology and Translational Medicine Texas A&M University College Station, TX 77843-1114
Email Address:	Parth.Khade@gmail.com
Education:	B.S., Biomedical Engineering Texas A&M University, May 2010 Undergraduate Research Scholar